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Breast Cancer

PRINCIPAL INVESTIGATOR: Nicoletta Eliopoulos, Ph.D.

CONTRACTING ORGANIZATION: McGill University  
Montreal, Quebec, Canada H3A 2T5

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13. ABSTRACT (Maximum 200 Words) Breast cancer is the most common malignancy in women. When the cancer remains localized, long-term survival is possible following surgery to remove the primary tumor. However, when the cancer metastasizes, death occurs for most patients. The progression and metastasis of breast cancer is dependent on angiogenesis. Tumors continuously dispense angiogenic signals. One such signal is erythropoietin (Epo). The general idea of our gene therapy approach is that breast cancer cells can be genetically engineered to release soluble Epo receptor (sEpoR), an antagonist to breast cancer-associated Epo and thus antagonist to breast cancer-associated angiogenesis. We presume that interfering in this manner with tumor-associated blood vessels should bring about a significant anti-tumor effect. Women die of breast cancer despite undergoing surgery, radiotherapy and chemotherapy. These women for whom all else fails may benefit from the novel therapeutic tactic that we propose due to the fact that the majority of women who succumb from breast cancer have metastatic or unresectable cancer that is refractory to standard treatment. Furthermore, the target of our strategy is tumor-associated neovasculature, which is not of malignant origin. Therefore, it is unlikely that these vascular cells have acquired "resistance to treatment", and they should remain responsive to antagonistic stimuli.			
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**Annual Summary Report for Concept Award BC023328 "Soluble Erythropoietin Receptor for Gene Therapy of Breast Cancer" DAMD17-03-1-0694**

## **INTRODUCTION**

Erythropoietin (Epo), an essential cytokine for erythropoiesis, acts through a membrane-bound Epo receptor (EpoR). A soluble form of this EpoR, namely sEpoR, is thought to function in regulating Epo levels via Epo binding and consequently causing ligand inactivation. Epo is also involved in angiogenesis, a required process in cancer growth and metastasis, and in augmenting human vascular endothelial growth factor. Endogenous or exogenous Epo can stimulate EpoR expressing cancer growth and persistence (Acs G et al. *Cancer Research* 61: 3561-3565, 2001). Analysis of 50 clinical breast carcinoma specimens revealed expression of Epo and Epo-R which were associated with malignant cells and tumor vasculature (Acs G. et al. *Cancer Research* 61: 3561-3565, 2001). Levels of expression were greatest in hypoxic tumor areas suggesting a role of Epo signaling in hypoxic survival of neoplastic cells. A further investigation showed that the increased Epo levels and consequently Epo signaling promotes survival of cancer cells and cancer-associated endothelial cells (Sasaki R et al., *Carcinogenesis* 23: 1797-1805, 2002). In this study, a dose-dependent reduction in malignant cells and capillaries via induction of apoptosis was noted subsequent to *in vitro* injection of anti-Epo monoclonal antibody or of sEpoR into blocks of breast tumor specimen. An *in vivo* study suggested the functional importance of Epo and EpoR in a rat model of breast cancer whereby a reduction in tumor progression was achieved through administration of anti-Epo antibody, sEpoR, or Jak2 kinase inhibitor (Arcasoy M.O. et al., *Laboratory Investigation* 82: 911-918, 2002).

## **RATIONALE AND OBJECTIVES**

While the above studies have demonstrated the potential of recombinant proteins such as sEpoR, it is conceivable that any lasting anti-tumor effect *in vivo* would necessitate repeated incommodeous injections of these agents. We thus hypothesize that a gene therapy strategy utilizing sEpoR to interfere with Epo signaling may lead to a greater and more sustained therapeutic action against Epo-expressing breast neoplasms. We have developed a robust retrovirus-based gene transfer technology that allows for efficient *in vivo* cancer-targeted gene delivery. This technological platform (Eliopoulos N. et al., *Gene Therapy*, 10: 478-489, 2003) will allow us to gene engineer cancer cells to express sEpoR. Therefore, we propose a proof of principle study where Epo-expressing breast cancer cells will be genetically engineered to secrete biologically relevant amounts sEpoR for anti-tumor and anti-angiogenic gene therapy of breast cancer. The main objectives are the following: (a) Retrovectors encoding for sEpoR will be generated, utilized to transduce breast cancer cell lines and production of sEpoR characterized *in vitro*. (b) Mice will be implanted with sEpoR engineered cancer cells and utilized to evaluate the main hypothesis of biological effect of sEpoR engineered breast cancer cells on tumor growth and vasculature *in vivo*.

## **PROPOSED METHODS**

The sEpoR cDNA, generously provided by Dr. Y. Nakamura, will be incorporated in a retroviral vector that co-expresses the green fluorescent protein (GFP) reporter. Retroparticles generated will be used to transduce human breast cancer cell lines BT-549 and MCF-7, provided by American Type Culture Collection (ATCC), with reported basal and hypoxia-induced expression of Epo and EpoR (Acs G. et al. *Cancer Research* 61: 3561-3565, 2001). Gene transfer efficiency will be assessed by GFP reporter expression through flow cytometry, fluorescent microscopy and histochemistry. Retrovector stability will

be determined by Southern and Northern blot analysis of gene-modified cells. Secretion of sEpoR by engineered breast cancer cells will be measured by ELISA *in vitro* (conditioned media). Functional anti-Epo activity of recombinant sEpoR secreted by transduced cells will be analyzed by the ability to bind to human  $^{125}\text{I}$ -Epo, and as a competitive inhibitor of Epo-induced proliferation of human breast cancer and human umbilical vein endothelial cells *in vitro*. As a murine model of breast cancer with increased Epo signalling, we will utilize the BT-549 and MCF-7 human mammary carcinomas in Non-Obese Diabetic / Severe Combined Immunodeficient (NOD/SCID) mice. Cells transduced with recombinant sEpoR will be implanted subcutaneously into mice. The effect of sEpoR secreting cells on tumor growth will be examined and compared to controls (non-transduced tumor, control vector transduced tumor). Biochemical protein assay on mouse plasma will determine if detectable transgene expression occurs and will be correlated with tumor response (if it occurs). *In vivo* plasma levels of recombinant sEpoR will be measured by ELISA. Immunohistochemical analysis will be performed on deparaffinized slides of tumor sections. Correlative studies examining tumor growth, vessel density and expression of angiogenic factors will be performed as we have previously described (Al-Khalidi A. et al. *Gene Therapy* 10(8): 621-629, 2003). Therapeutic effect as measured by tumor regression and animal survival will be assessed.

## RESULTS and KEY RESEARCH ACCOMPLISHMENTS

Several of the major objectives of Task 1 of the Statement of Work have been completed, i.e.

Task 1. *Production and characterization of a novel retroviral vector comprising the human soluble Erythropoietin receptor (sEpoR), Months 1-4:*

Specifically, with regards to the aims of Task 1 of the Statement of Work shown below in italic font,

- a. *Constructs:* We have already obtained the human sEpoR cDNA from Dr. Yukio Nakamura, from the BioResource Center of RIKEN Tsukuba Institute in Japan.  
We received the vector “pCDM8” containing the human sEpoR cDNA.
- b. *Clone the human sEpoR cDNA into our previously published retrovector construct co-expressing the green fluorescent protein (GFP) and generate replication-free retroparticles.*

We removed the human sEpoR cDNA, by restriction enzyme digest, from the pCDM8 vector kindly provided by Dr. Nakamura. We then successfully, although only after considerable technical difficulties, cloned this sEpoR cDNA into the commercially available expression vector pBluescriptKS. The resulting pBluescriptKS-sEpoR plasmid construct was utilized to conduct preliminary studies, and also served as a shuttle vector to more easily introduce the sEpoR cDNA from it into our published retrovector AP2 expressing the reporter green fluorescent protein. The reason for first introducing the sEpoR cDNA into pBluescriptKS instead of introducing it directly into AP2 was to obtain more possible restriction sites surrounding the cDNA for sEpoR to thereafter more theoretically feasibly introduce this sEpoR cDNA into AP2. More specifically, the cloning strategy was the following: 1) Retrieve by XbaI digest the sEpoR cDNA from pCDM8 provided to us by Dr. Nakamura, 2) Introduce the sEpoR cDNA flanked by 2 XbaI sites into the XbaI site present in the multiple cloning site of pBluescript KS, thus generating pBluescriptKS-sEpoR, 3) Retrieve by EcoRI and SalI digest the sEpoR cDNA from pBluescriptKS-sEpoR and introduce it into the BamH1/Klenow and XbaI (compatible with SalI) digested AP2, thus generating AP2-sEpoR. Before completing the entire cloning process and conducting experiments with the resulting AP2-sEpoR, we decided to commence analysis using the pBluescriptKS-sEpoR. At time of

submission of the first annual report, we were in the process of verifying, through restriction enzyme analysis and subsequently through sequencing, that this cloning of the sEpoR cDNA into the AP2 retrovector had been successful and without the introduction of any sequence errors. At the time now of submission of this revised first annual report, we have completed the restriction enzyme analysis and sequence analysis, and have not noted any sequence errors.

- c. *Transduce human breast cancer cell lines (BT-549 and MCF-7 from American Type Culture Collection), and determine gene transfer efficiency, vector stability, transgene expression, and sEpoR protein secretion levels.*

As indicated above, we were in the process of verifying that the cloning of the sEpoR cDNA into the AP2 retrovector was error-free, and thus have not yet generating retroviral particles containing the human sEpoR for transduction of the BT-549 and MCF-7 human breast cancer cell lines from *American Type Culture Collection* (ATCC). However, since in our cloning strategy we first generated the plasmid construct pBluescriptKS-sEpoR prior to the retroviral construct AP2-sEpoR, we commenced preliminary studies to first test our hypothesis in transfected prior to retrovirally transduced cell lines. We ordered the BT-549 and MCF-7 cell lines from ATCC but due to the poor state of the cells received, ATCC kindly sent us a new shipment of these cells without cost, since also encountered by other clients, but adding to unforeseen experimental delays. After expanding and being confident about the quality of the new BT-549 and MCF-7 cells, we then transfected these breast cancer cell lines each with 10ug of pBluescriptKS-sEpoR and 1ug of pEGFP-N1 plasmid conferring neomycin resistance. The pEGFP-N1 plasmid was used in this co-transfection and at a 10-fold lower amount than pBluescriptKS-sEpoR to allow for drug selection of sEpoR gene-modified cells, more specifically of cells taking up both pBluescriptKS-sEpoR and pEGFP-N1, since neomycin resistance is provided by pEGFP-N1. Drug selection was also necessary because unlike the subsequently generated construct AP2-sEpoR, the GFP reporter is not present in pBluescriptKS-sEpoR. Following drug selection of pBluescriptKS-sEpoR and pEGFP-N1 transfected cells, we obtained the sEpoR transfected BT-549 and sEpoR transfected MCF-7 cells. As a non-breast cancer control, we also likewise generated sEpoR transfected A549 human lung cancer cells. In addition, as non-sEpoR controls, we similarly transfected BT-549, MCF-7, and A549 cells with the control plasmid, i.e. pBluescriptKS which does not contain the sEpoR cDNA.

Due to the technical difficulties and unforeseen delays that occurred while attempting to complete the objectives of Task 1, we have not yet carried out the objectives of Task 2 of the Statement of Work

*Task 2: Determination of the therapeutic efficacy of transplanted sEpoR gene-modified human breast cancer cells in vivo, Months 5-12:*

Specifically, the aims of Task 2 of the Statement of Work are the following:

- a. *Implant Non-Obese Diabetic / Severe Combined Immunodeficient (NOD/SCID) mice (over 30 with controls) with sEpoR gene-modified human breast cancer cell line BT-549, control vector engineered BT-549 cells, or non-modified BT-549 cells.*

- b. Likewise, in order to assess effect in another relevant mammary carcinoma, implant NOD/SCID mice (over 30 with controls) with sEpoR gene-modified human breast cancer cell line MCF-7, control vector engineered MCF-7 cells, or non-modified MCF-7 cells.
- c. Monitor tumor growth and correlate with sEpoR transgene expression assessed by biochemical protein assay on plasma. Determine also expression of other angiogenic factors, such as vascular endothelial growth factor.
- d. Perform immunohistochemical analysis on tumor sections examining vascular and related structures from all groups of mice.
- e. Monitor impact of sEpoR expression on normal erythropoiesis by serial measurement of hematocrit over time in test mice.

We will conduct hematocrit measurements over time for all groups of mice, tests i.e. mice with sEpoR modified cancer cells, and controls i.e. mice with control vector (no sEpoR) modified cancer cells, and mice with unmodified cancer cells. We do not expect to see a change in hematocrit but will assess and address this possibility. In case of an effect on hematocrit in test mice, we will ascertain if there is a dose-effect and also determine if the dose of cells leading to no hematocrit change has an antitumor effect. Initial measurement of antitumor effect will be serial measurement of tumor size over time. An effect of any anemia on tumor response is a theoretical concern but not an expected result. In case of this unexpected result occurring, we would also include supplementary control groups in experiments devised to determine the effect of anemia on its own.

## CONCLUSION

This proposal is novel as none has yet explored local delivery of sEpoR for gene therapy of cancer, breast cancer in particular.

We initially encountered experimental obstacles and are thus now grateful to have received a 1-year, no-cost extension, as seen below, on this Concept Award proposal entitled "*Soluble Erythropoietin Receptor for Gene Therapy of Breast Cancer*", Log Number BC023328, Grant Number DAMD17-03-1-0694. As indicated in the e-mail we sent to Dr. Kimbark, the unforeseen technical difficulties and experimental delays, such as related to cloning of the sEpoR cDNA into our vector, generated the need to ask if the award period can be extended, without extra funds, to allow the completion of the proposed work. We thereafter were pleased to receive the following document sent by Sherry M. Apperson, Procurement Technician, United States Army Medical Research Acquisition Activity, Customer Service Center- Blue Team.